Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal for Parasitology 35 (2005) 1407–1415
www.elsevier.com/locate/ijpara

Immunization with native surface protein NcSRS2 induces a Th2 immune response and reduces congenital *Neospora caninum* transmission in mice ☆

G.J. Haldorson[a], B.A. Mathison[a], K. Wenberg[a], P.A. Conrad[b], J.P. Dubey[c], A.J. Trees[d], I. Yamane[e], T.V. Baszler[a,*]

[a]*Department of Veterinary Microbiology and Pathology, Washington State University, Bustad Hall, Pullman, WA 99164-7040, USA*
[b]*Department of Veterinary Microbiology and Immunology, University of California, Davis, CA 95616, USA*
[c]*Animal Parasitic Disease Laboratory, United States Department of Agriculture, Agricultural Research Services, Beltsville, MD 20705-2350, USA*
[d]*Veterinary Parasitology, Liverpool School of Tropical Medicine and Faculty of Veterinary Science, University of Liverpool, Liverpool L35QA, UK*
[e]*National Institute of Animal Health, 3-1-1 Kannondai, Tsukuba-shi, Ibaraki Japan 305*

Received 11 March 2005; received in revised form 20 May 2005; accepted 20 May 2005

Abstract

NcSRS2, a tachyzoite surface protein of *Neospora caninum*, is an immunodominant protein with respect to induction of antibody production and has a role in attachment and invasion of host cells. Native NcSRS2 was isolated from whole tachyzoite lysate antigen by affinity chromatography using NcSRS2 specific monoclonal antibody and used to immunize BALB/c mice in a congenital transmission study. NcSRS2 was a highly conserved protein as indicated by comparison of deduced amino acid sequence obtained from NcSRS2 gene sequences of 10 geographically distinct *N. caninum* isolates. Mice immunized with purified native NcSRS2 produced antigen-specific antibody, primarily of IgG 1 subtype. Following challenge during gestation with 10^7 tachyzoites, immunized mice had a statistically significant decreased frequency of congenital transmission compared to non-immunized mice ($P \leq 0.05$) or mice inoculated with adjuvant alone ($P \leq 0.01$). Decreased congenital transmission among immunized mice correlated with a predominately Th2 immune response compared to non-immunized mice as indicated by an increased ratio of interleukin 4 (IL-4) to interferon gamma (IFN-γ) secretion from antigen-stimulated splenocytes. The results provide a rationale for NcSRS2 as a candidate subunit vaccine antigen for reduction of *N. caninum* congenital transmission. Furthermore, the studies suggest that a Th2 immune response, if directed against an appropriate antigen, may induce protection against *N. caninum* congenital infection in mice.

Keywords: *Neospora caninum*; Mouse; Immunity; Congenital transmission; SRS2

1. Introduction

Neospora caninum is an apicomplexan parasite closely related to *Toxoplasma gondii*. The parasite infects a wide rage of species, though to date, the only known definitive hosts for *N. caninum* are domestic dogs (McAllister et al., 1998) and coyotes (Gondim et al., 2004). While many mammalian species can be infected based upon evidence of seroconversion, clinical disease occurs primarily in dogs and cattle. In cattle, clinical disease is manifested as parasite-induced abortions (Dubey, 2003). Congenital transmission, resulting in a clinically normal but infected calf, occurs and is likely the most common means of perpetuation of infection within a given herd (Dubey, 2003). Therefore, development of an effective vaccine to prevent congenital transmission would prove beneficial for control of neosporosis in cattle herds.

Research towards development of effective vaccines for the prevention of neosporosis to date shows mixed results. Clearly, immunity to fetal infection and abortion induced by *N. caninum* can occur in experimentally and naturally

☆ Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers AY940480, AY940481, AY949482, AY940483, AY940484, AY940485, AY940486, AY940487, and AY940488.

* Corresponding author. Tel.: +1 509 335 6047; fax: +1 509 335 8529.
E-mail address: baszlert@vetmed.wsu.edu (T.V. Baszler).

0020-7519/$30.00 © 2005 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2005.05.013

infected cattle (Innes et al., 2001; Williams et al., 2003). Immunity to experimental neosporosis in mice is associated with a T helper 1 (Th1) immune response dominated by production of interferon gamma (IFN-γ) and IgG2a antibody induction and a similar response may be protective in cattle (Khan et al., 1997; Long et al., 1998; Baszler et al., 1999). Immunization of mice with various recombinant surface and dense granule proteins has demonstrated protection against systemic disease (Cho et al., 2005). In addition, immunization of mice with whole killed tachyzoites followed by experimental infection results in reduction of congenital transmission but the mechanism of immunity is unknown (Liddell et al., 1999). However, in an encephalitis model in mice, immunization with tachyzoite sonicate can result in exacerbation of disease suggesting that identification of specific protection-inducing antigens may be important (Baszler et al., 2000). In ruminants, evaluation of whole *N. caninum* tachyzoite protein vaccines shows similar equivocal results. A study in 2000 showed that vaccination of cattle with a whole tachyzoite antigen did not reduce congenital transmission rates following subsequent experimental challenge (Andrianarivo et al., 2000). A later study with a commercially available whole tachyzoite vaccine under field conditions demonstrated a reduction in general abortion rates in vaccinated cattle, although precise causes of the abortions and adjuvant effects were not determined (Romero et al., 2004). In sheep, one study using whole tachyzoite immunizations detected no change in abortion rate, but reduced congenital transmission (O'Handley et al., 2003), while a second study resulted in improved fetal survival, but no effect on congenital transmission rate (Jenkins et al., 2004).

Methods for improvement of *N. caninum* vaccines may require subunits that direct the immune system toward specific antigens or epitopes that induce a protective response. In this regard, much research is directed toward *N. caninum* surface proteins in attempt to target the immune responses toward antigens accessible during the extracellular phase of parasitemia, or toward proteins crucial for parasite transmission and survival. One of these surface proteins, *N. caninum* SAG1 related sequence 2 (NcSRS2) has previously been shown to have a role in attachment and invasion of host cells (Nishikawa et al., 2000a). Most work with NcSRS2 as an immunogen has focused upon DNA vaccines and/or recombinant proteins. Nishikawa and coworkers (Nishikawa et al., 2000b) have developed recombinant vaccinia virus vectors that express the NcSRS2 gene. Immunization with the vaccinia virus that expresses NcSRS2 resulted in reduced parasite load in tissues following challenge compared to animals immunized with vector alone (Nishikawa et al., 2001a). Similarly, immunization with this recombinant virus resulted in reduction in congenital transmission in females vaccinated before pregnancy and challenged during pregnancy (Nishikawa et al., 2001b). In a cerebral infection model, immunization with recombinant NcSRS2 resulted in production of antibodies to recombinant, but not native protein, and only slight reduction in infection relative to adjuvant controls. However, when recombinant NcSRS2 was combined with the eukaryotic expression plasmid pcDNA3 containing a NcSRS2 cDNA insert, antibodies to both recombinant and native protein were produced and cerebral infection following subsequent challenge was significantly reduced (Cannas et al., 2003). These findings suggest that protein in its native state may be required to induce an effective immune response.

The purpose of the current work is to determine whether or not native NcSRS2 would induce protective immunity to congenital neosporosis and to correlate that protection to a specific immune response. The results indicate that immunization of mice with native NcSRS2 prior to pregnancy induced protective immunity to parasite challenge given during pregnancy. Surprisingly, protective immunity correlated with a Th2 immune response.

2. Materials and methods

2.1. NcSRS2 sequence comparison

2.1.1. Parasites

Ten geographically distinct *N. caninum* isolates obtained from naturally infected hosts were used as a source of NcSRS-2 DNA for sequence comparison. Sources of isolates were as follows: BPA1, bovine isolate from the USA (Conrad et al., 1993); BPA6, bovine isolate from the USA (Barr et al., 1993); JPA4, bovine isolate from Japan (Yamane et al., 1998); JPA5, bovine isolate from Japan (Yamane et al., 1998); NC1, canine isolate from the USA (Dubey et al., 1988); NC2C, canine isolate from the USA (Hay et al., 1990); NC5, canine isolate from the USA (Dubey et al., 1988); NCLiv, canine isolate from the UK (Barber et al., 1995); SweB1, bovine isolate from Sweden (Stenlund et al., 1997); and DubeyB1 (NC-beef1), bovine isolate from the USA (McAllister et al., 2000). The Dubey B1 isolate (also known as NC-beef1) was isolated in the laboratory of one of us (JPD) in equine dermal cells inoculated on February 28, 1998 with brain homogenate of a beef calf that was born prematurely. Tachyzoites were first seen in the flask 31 days later and subpassaged to new flasks at that time. Thus, this strain had been cultured in vitro for only a few passages before use in the present study. For all isolates, parasite tachyzoites were propagated in Vero cells using Dulbeco's modified essential medium (DMEM), 10% fetal calf serum (FCS), at 37 °C in 95% air/5% CO_2 atmosphere. Vero cell cultures were grown to confluency and infected with *N. caninum* and allowed to lyse. Separation of parasites from Vero cell debris was accomplished by passage through 10 μm nylon filters (Millipore). The parasites were washed several times in PBS and centrifuged at 800 g for 20 min. The pellets were stored at −20 °C.

2.1.2. Isolation of parasite DNA and PCR amplification of NcSRS-2 genes

Thawed pellets of tachyzoites were incubated overnight in lysis buffer (50 mM Tris–HCl (pH 8), 100 mM EDTA, 100 mM NaCl,1% SDS) and 200 μg/ml Proteinase K at 55 °C (Long and Baszler, 2000). DNA isolation was performed by the Phenol:chloroform:isoamyl alcohol method and precipitated in ethanol (Burns et al., 1997). The pellet was stored at −86 °C. The NcSRS-2 gene fragments were obtained by PCR using forward primer SRS-2F (5′ATGGCGACGCATGCTTGTGTGGTG3′), and reverse primer SRS-2R (5′GTACGCAAAGATTGC CGTTGC3′) (Marsh et al., 1999). The PCR conditions were (94 °C for 1 min) 35 cycles (94 °C for 30 s, 68 °C for 3 min, 68 °C for 3 min) and an extension (15 °C for 7 min). The amplicons were identified by gel electrophoresis (agarose 1%) at 100 V followed by staining with ethidium bromide.

2.1.3. Cloning and sequencing

The amplified PCR products were cloned using a TOPO TA cloning kit (Invitrogen). Several colonies were selected of each isolate for sequencing. Plasmid DNA was isolated using the Wizard Plus SV Miniprep DNA purification system (Promega) and the sequencing reaction employed the dye termination method. Both strands were sequenced using an ABI 3100 Genetic Analyser.

2.1.4. Analysing DNA sequences

Results of sequencing reactions were analyzed using DNA Star's SEQman version 5.0. Five to 10 clones per isolate were sequenced to obtain a reliable consensus sequence. Sequences were aligned and a consensus sequence was produced for each of the 10 isolates. The consensus sequences were aligned using the CLUSTALW method and NcSRS-2 gene and deduced amino acid sequences compared between isolates and published sequences.

2.2. Isolation of native NcSRS2

2.2.1. Development of NcSRS2-specific antibody and affinity column

Surface proteins in their native conformation were isolated using NcSRS2-specific mAb 100.2.4 loaded on a cyanogen bromide-activated Sepharose 4b immunoaffinity column similar to previously described methods (Debard et al., 1996). The hybridoma secreting mAb 100.2.4 was generated from splenocytes of a mouse infected with live *N. caninum* tachyzoites using standard methods previously described in our laboratory (Baszler et al., 1996). The antibody was shown to recognize a diffusely distributed surface antigen by indirect fluorescent antibody test (data not shown). In short, tachyzoites were attached to glass slides using a Cytospin® cytocentrifuge followed by fixation with acetone and air drying. The glass slides were then incubated with mAb 100.2.4 for 1 h at room temperature, washed with PBS, incubated with fluorescein conjugated goat anti-mouse Ig for 1 h at room temperature in the dark, and washed with PBS again. The slides were then examined under fluorescent light.

Approximately 76 mg of mAb 100.2.4 was incubated with cyanogen bromide-activated Sepharose 4b overnight followed by blocking with 0.3 M Tris (pH 7.4) and alternating pH 4 and pH 8 washes. Binding of greater than 95% of antibody to the Sepharose was confirmed by comparisons of OD_{280} before and after exposure to the gel. Following washing and resuspension in PBS, the Sepharose with bound mAb was then added to a laboratory column.

2.2.2. Affinity purification and characterization of native NcSRS2

The NC-1 strain of *N. caninum* parasites were grown in Vero cell cultures and collected when 90–95% of the Vero cells had ruptured. Tachyzoites were washed three times in PBS then resuspended in detergent (1% NP40, Boering Mannheim) and protease inhibitor (1 mM phenylmethylsulfonylfluoride, Spectrum Quality Products, Inc.) in water. The resuspended tachyzoites were sonicated at full power for six pulses of 30 s each. The sonicate was centrifuged at 800 g for 20 min at 4 °C and the supernatant (Nso) was collected for affinity chromatography. Crude protein (Nso) was then passed over the column, the column was washed, and purified protein was eluted with 3 M NaSCN.

The purity of the protein was analysed by SDS-PAGE and Western blot. For each, 20 μg of antigen (Nso or column eluent) was loaded per lane in pre cast, 4–20% gradient acrylamide gels (Biorad). Following electrophoresis, one gel was fixed in 30% ethanol and 10% acetic acid, washed and incubated with 0.1% silver nitrate. Silver staining was then identified by incubating the gel with 2.5% sodium carbonate and 0.02% formaldehyde and stopped with 1% acetic acid. Proteins on the second gel were transferred to a nitrocellulose membrane using standard methods. Prior to immunodetection, the membrane was blocked with 2.5% milk in PBS with 0.05% Tween 20. Following blocking, the membrane was divided into thirds and probed with anti-*Neospora* antiserum, mAb 100.2.4, or no primary antibody. Bound antibody was detected by chemiluminescence using HRPO-conjugated, goat anti-mouse IgG and IgM followed by tetramethylbenzidine (TMB, Sigma) and exposure of radiographic film.

To further confirm purity and to ensure that the purified native NcSRS2 stimulated antibody production in the mouse, 20 μg of eluted protein suspended in Freund's incomplete adjuvant (total volume 200 μl) was injected subcutaneously three times at 10 day intervals into four mice. Serum was collected from each mouse prior to the first immunization and following each of the immunizations. The serum from each collection was evaluated by ELISA (using native affinity purified NcSRS2 as antigen) and by western blot loaded with whole tachyzoite antigen (Nso).

2.3. Experimental design of NcSRS2 immunization and challenge

All animal use was approved by the Washington State University Institutional Animal Care and Use Committee. The timeline for immunization, challenge infection, and detection of congenital transmission is outlined in Fig. 1. Eight to 10 week old female BALB/c mice were divided into three groups of 15–20 mice each. Group 1 consisted of 15 untreated mice (congenital transmission control). Group 2 included 15 mice that were injected with 200 μl of a 50:50 mixture of PBS and Freund's incomplete adjuvant (adjuvant control). Group 3 was the treatment group and included 20 mice that were immunized with 20 μg of native NcSRS2 in Freund's incomplete adjuvant (200 μl total volume). Each injection in the mice in groups 2 and 3 was given subcutaneously four times at 10 day intervals, with the last injection occurring 7 days prior to exposure to males.

Each individual female mouse was then housed for 5 days with a 10–12 week old male mouse that had previously sired a litter. Females were examined three times daily for evidence of a copulatory plug. The first day that a copulatory plug was seen was designated as day 0 of pregnancy. On day 10 of pregnancy, each female mouse was challenged with 1×10^7 NC-1 tachyzoites i.p. At 7 days of age, mouse pups were euthanized and brain and lungs were collected using individual sterile instruments and stored at −80 °C for subsequent PCR evaluation of infection. At the same time, the dams were euthanized for collection of cardiac blood for serum antibody analysis and spleens for splenocyte cytokine analysis. Spleen cells were isolated by triturating in DMEM using a 21-gauge needle and the single cell suspension of splenocytes suspended in 90% serum and 10% DMSO and frozen at −80 °C.

2.4. Detection of congenital transmission by PCR

DNA was extracted from a total of 25 mg of brain and lung from each pup using a DNeasy® kit (Qiagen). A semi-nested PCR was then performed on the pup DNA using primers NP6/NP21 amplifying a 321 bp oligonucleotide followed by NP6/NP7 which amplifies a 220 bp fragment as previously described (Yamage et al., 1996). The entire process was repeated once for each mouse pup. Controls included tachyzoite-spiked mouse tissue as the positive control, and negative mouse pup tissue (pups from normal, non-infected dams) and water only as the negative controls. In our laboratory the nested PCR using the primer pairs described above has a detection limit of one tachyzoite per 15 mg of tissue (data not shown). The congenital infection rate or frequency of transmission was the number of PCR positive pups divided by the total number of pups for that respective dam. The mean congenital transmission rate per dam was calculated and differences between NcSRS2 vaccinated and control groups compared by Chi Square analysis ($P\leq0.05$).

2.5. Antibody isotyping

Serum from terminal cardiac blood collection of dams was used to measure antigen-specific IgG1 and IgG2a titers by ELISA as described previously (Baszler et al., 1999). Native NcSRS2 was substituted for whole tachyzoite antigen (Nso) as antigen.

2.6. Cytokine profile

Parasite-specific IL-4 and IFN-γ secretion was measured by ELISA as previously described except native NcSRS2 was used for antigen-specific in vitro stimulation (Baszler et al., 1999). Each well was cultured for 3 days followed by 24 h of stimulation. Five wells were incubated for each splenocyte preparation including one well loaded with 5 μg of ConA (positive control), one well with no antigen added (negative control) and three wells incubated with 9 μg per well each of native NcSRS2. The O.D. of each well was determined by an electronic plate reader at a wavelength of 450 nm and converted to ng/ml using a standard curve generated using serial dilutions of known concentrations of rIL-4 and rIFN-γ (R&S Systems Inc., Minneapolis, MN, USA). The mean cytokine level and SD and IL-4:IFN-γ ratio and SD of NcSRS2-immunized and control groups were calculated and groups compared by ANOVA ($P\leq0.05$).

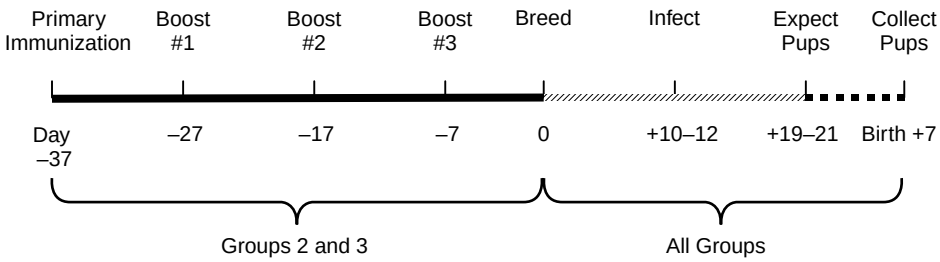

Fig. 1. Timeline of treatments for NcSRS2 immunization, challenge infection with *Neospora caninum* tachyzoites, and detection of *N. caninum* congenital transmission by PCR.

3. Results

3.1. NcSRS2 sequence comparison

NcSRS2 genes generated by PCR from each geographical isolate of *N. caninum* described in materials and methods were successfully cloned and sequenced. Combining five to 10 sequences of each clone generated a consensus sequence for each isolate. Differences in single nucleotides between clone sequences from individual isolates were resolved using the majority rule. Alignment of the consensus sequences from all 10 isolates using the ClustalW method revealed 99.7–100% NcSRS2 sequence identify at the gene and deduced amino acid sequence level. Isolates BPA1, BPA6, JPA4, NC1, NC2C, NC5, NCLiv, SweB1, and Dubey B1 had 100% sequence identity of NcSRS2. A single base difference was observed only for the JPA-5 consensus sequence. The translated protein differed in a single amino acid at position 338 where the JPA-5 translated a glutamine when the other nine isolates translated a lysine.

3.2. Isolation and characterization of affinity purified native NcSRS2

Monoclonal antibody 100.2.4 was identified as IgG2a and bound diffusely to the exterior surface of viable *N. caninum* tachyzoites as indicated by IFA (data not shown). Dotblot assay revealed binding of mAb 100.2.4 at low concentrations (0.05 ug/ml) to native sonicated *N. caninum* antigen (Nso) (not shown). Western blotting analysis (Fig. 2b) revealed mAb 100.2.4 bound to a single *N. caninum* tachyzoite antigen with a molecular mass of 42 kDa (Fig. 2b, lane 3); an antigen of the same size was bound by polyclonal *N. caninum* positive mouse serum (Fig. 2b, lane 1).

The relative purity of antigen collected from the affinity column was demonstrated by SDS-PAGE and Western blot analysis (Fig. 2a and b). Based upon the SDS-PAGE under reducing conditions, three major molecules of molecular mass 25, 42, and 50 kDa were isolated following elution of the 100.2.4 affinity column (Fig. 2a). Three antigens of similar molecular masses were recognized by western blot analysis of column eluates when probed with either mouse polyclonal anti-*Neospora* serum or with mAb 100.2.4 (Fig. 2b, Lanes 2 and 4). A control consisting of incubation with only the anti-mouse Ig secondary antibody showed strong reactivity of the 25 and 50 kDa proteins, but not the 42 kDa protein, indicating that the 25 and 50 kDa bands represented the heavy and light chains of mouse immunoglobulin and that the 42 kDa protein was *Neospora* specific (Fig. 4B, Lane 6).

To further characterize the 42 kDa antigen in column eluates, two BALB/c mice were immunized with the column eluate antigen preparation as described in the Materials and methods. Western blot analysis of whole tachyzoite antigen Nso probed with sera from immunized

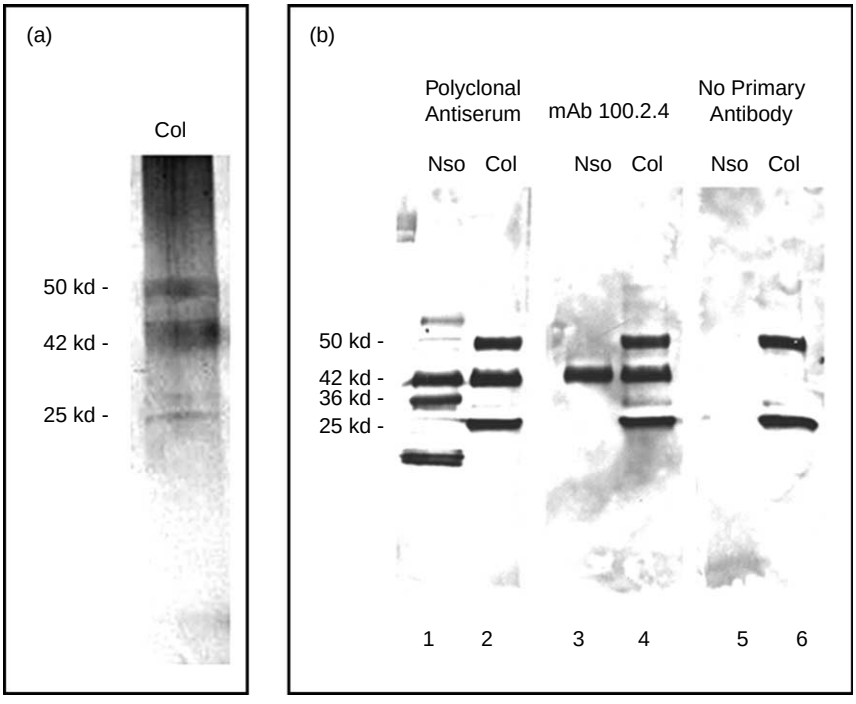

Fig. 2. SDS-PAGE and Western blot analysis of *Neospora caninum* tachyzoite antigen (Nso) and affinity column purified NcSRS2. (a) SDS-PAGE of NcSRS2 affinity column eluate (4–20% gradient gel). The position of molecular mass standards is indicated on the left. (b) Western blot of *N. caninum* tachyzoite antigen (Nso) (Lanes 1, 3, 5) or NcSRS2 affinity column eluate (Lanes 2, 4, 6) probed with polyclonal mouse anti-*Neospora* serum at 1:1,000 (Panel 1), mAb 100.2.4 at 8 ug/ml (Panel 2), or buffer alone as primary antibody (negative control) (Panel 3). The position of molecular mass standards is indicated on the left.

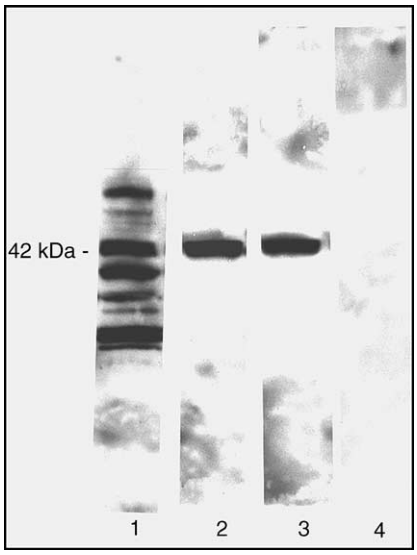

Fig. 3. Western blot analysis of serum from *BALB/c* mice immunized with affinity column purified native NcSRS2. *Neospora caninum* whole tachyzoite sonicate (Nso) was used as antigen and probed with polyclonal mouse anti-*Neospora* serum (lane 1), NcSRS2-specific mAb 100.2.4 as positive control (lane 2), mono-specific anti-serum from NcSRS2 immunized mice (lane 3), or non-immune serum from naïve mouse (lane 4). Molecular mass indicator of NcSRS2 indicated on left.

mice revealed immunoreactivity of a single 42 kDa band (Fig. 3, Lane 3), similar to Nso probed with anti-NcSRS2-specific mAb 100.2.4 (Fig. 3, lane 2).

3.3. Analysis of pregnant BALB/c mice immunized with native NcSRS2

Results of the congenital transmission studies are summarized in Table 1. NcSRS2-immunized mice (Group 3) had decreased frequency of transmission, both with respect to the percentage of dams transmitting and the total number of fetuses infected when compared with mice in control groups that were not immunized (Group 1) or inoculated with adjuvant only (Group 2). The 24% frequency of fetal transmission of parasites in the NcSRS2 immunized group was significantly lower when compared with the 45% congenital transmission rate non-immunized control group (Chi square 3.91, $P<0.05$) and the 62% congenital transmission rate in the group inoculated with adjuvant only (Chi square 6.96, $P<0.01$). The difference in the number of dams with congenital transmission was not statistically significant between groups (Group 1–7 of 7; Group 2–5 of 5; and Group 3–8 of 10). Multiple dams in each group of mice had pups that died on days 1 or 2 (seven to 10 total pups per group). In many cases, these pups were partially or completely cannibalized by their dams. Given that not all of these pups could be collected, results of PCR from their tissues were not included in the total number analyzed.

Immunized dams (Group 3) produced higher ($P<0.05$) levels of IL-4 (Fig. 4A) and lower ($P<0.01$) levels of IFN-γ (Fig. 4B) compared with non-immunized control mice (Group 1) and higher ($P<0.01$) levels of IL-4 compared to mice inoculated with adjuvant alone (Group 2). Taken together, there was a significantly higher IL-4: IFN-γ ratio in NcSRS2 immunized mice compared with both control groups (Fig. 4C) ($P\leq0.01$).

Immunization with native NcSRS2 in Freund's incomplete adjuvant induced the production of primarily parasite-specific IgG1 (mean IgG1 titer 1:1,200, mean IgG2a titer 1:100). Following challenge infection and gestation, although parasite-specific antibody titers increased in NcSRS2-immunized mice there was a predominant bias towards IgG1 isotype production, to the near exclusion of IgG2a (Mean IgG1 titer 1:4,000, mean IgG2a titer 1:70).

4. Discussion

The data presented indicate that immunization with affinity purified, native NcSRS2 can significantly reduce *N. caninum* congenital transmission in experimentally challenged BALB/c mice. Furthermore, protection in NcSRS2-immunized mice was associated with a Th2 immune

Table 1
Comparison of *Neospora caninum* congenital transmission between NcSRS2-immunized and control groups

Experimental groups	*N. caninum*-positive offspring/dam	Total offspring/dam		Frequency of transmission/dam
1. Non-immunized	1	5		0.20
	3	6		0.50
	2	4		0.50
	5	7		0.71
	1	4		0.25
	3	8		0.38
	2	4		0.50
Total	17	38	Mean	0.45
2. Adjuvant only	4	6		0.67
	3	4		0.75
	2	2		1.00
	1	6		0.17
	3	3		1.00
Total	13	21	Mean	0.62
3. NcSRS2-immunized	1	5		0.20
	1	6		0.17
	0	3		0.00
	1	2		.050
	3	3		1.00
	0	3		0.00
	1	6		0.17
	1	5		0.20
	1	5		0.20
	1	4		0.25
Total	10	42	Mean	0.24[a,b]

[a] Chi Square analysis with group 1, Chi Square = 3.91, $P<0.05$.
[b] Chi Square analysis with group 2, Chi Square = 6.96, $P<0.01$.

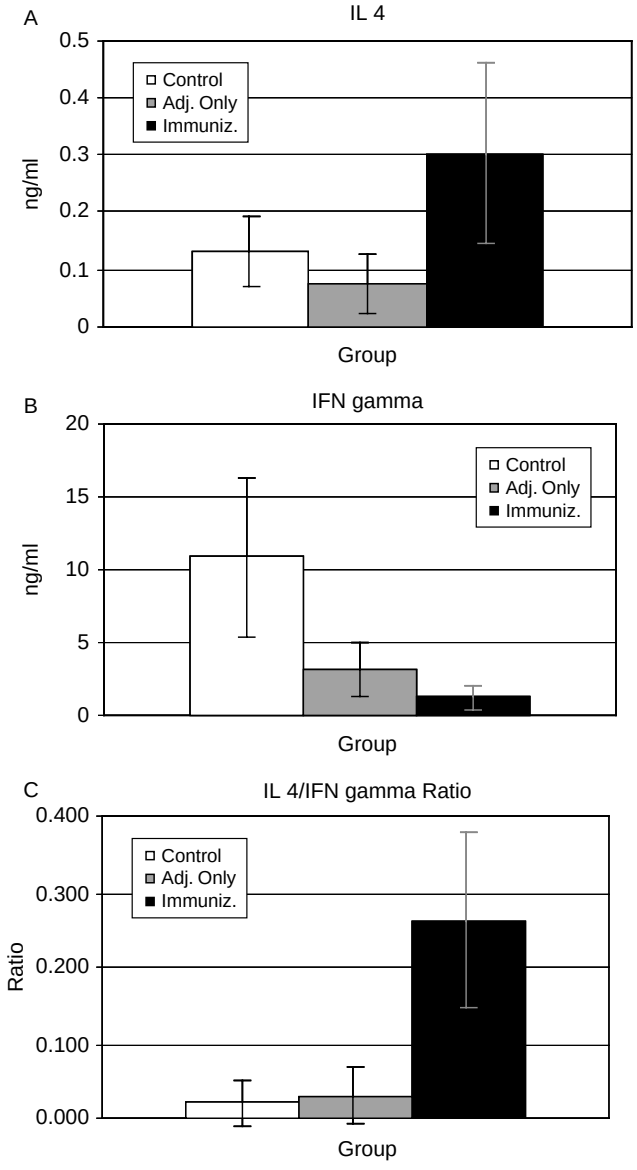

Fig. 4. Parasite-specific interleukin-4 (A) and interferon-γ (B) secretion or IL-4:IFN-γ ratio (C) from splenocytes stimulated in vitro with native NcSRS2. Splenocytes obtained from dams terminally after experimental treatment and challenge infection. Experimental treatments were: Group 1 = Non-immunized control. Group 2 = Adjuvant control (incomplete Freund's incomplete adjuvant only). Group 3 = Native NcSRS2 immunized with incomplete Freund's adjuvant. Bars = mean of group, error bars = 1 SD.

response as shown by parasite-specific splenocyte cytokine and serum antibody isotype analyses. To our knowledge, this is the first report of the potential efficacy of immunization with affinity purified, native NcSRS2 inducing a T helper 2 (Th2) immune response in a congenital transmission model. Vaccine development using native NcSRS2 would be desirable because it is a highly conserved parasite surface protein with potential for efficacy over diverse geographical regions worldwide.

The native NcSRS2 immunogen used in the experiments was affinity purified using anti-NcSRS2-specific monoclonal antibody 100.2.4. We confirmed the specificity of the NcSRS2 monoclonal antibody 100.2.4 several ways. Firstly, mAb 100.2.4 bound to a tachyzoite surface protein in its native state as shown by IFA. Secondly, both mAb 100.2.4 and anti-*N. caninum* polyclonal serum from *N. caninum*-infected mice bound a 42 kDa antigen, a molecular mass appropriate for NcSRS2 in western blot using whole tachyzoite lysate as antigen (Hemphill and Gottstein, 1996). Thirdly, in a separate experiment from our laboratory both anti-*N. caninum* polyclonal serum and mAb 100.2.4 bound a recombinant protein identical to the published sequence of NcSRS2, (Staska et al., 2005). It should be noted that reports of the size of NcSRS2 vary depending upon SDS-PAGE conditions. Some reports refer to a 35 kDa protein (under non-reducing conditions), while others refer to a molecular mass of 42 or 43 kDa (under reducing conditions), the latter similar to the studies reported herein (Hemphill, 1996; Howe et al., 1998). Most importantly, the relative purity of the antigen eluted from the affinity column was further confirmed in trial immunization studies, where mice immunized with affinity purified NcSRS2 produced monospecific antibodies to a single protein of the 42 kDa, the molecular mass for NcSRS2.

There were several critical observations made with the BALB/c congenital transmission model used in our study. The results may have been skewed some what by a relatively low percentage of pups surviving until day 7 of age. We attribute this finding, at least in part, by the high challenge dose that was given during pregnancy. The dose of 10^7 tachyzoites was based on previous research in our laboratory when pups were analyzed for infection at 1 day of age (Long and Baszler, 2000). Doses of 10^5 or 10^6 infectious organisms have been used in other published research to demonstrate *N. caninum* congenital transmission in mouse models when pups were analysed for infection at 7 days of age (Nishikawa et al., 2001b; Quinn et al., 2002). This 1–2 log difference in dose could explain reduced survival in all groups due to the challenge infection overwhelming the immune system. A second component was low fecundity of bred mice. In previous studies from our laboratory and others, congenital transmission could be expected to range between 80 and 100% rather than the 45% seen in our control group. The cause for low fecundity was not determined. However, these potential adverse components of the congenital transmission studies did not prevent demonstrating a statistically significant two- to three-fold reduction in *N. caninum* congenital transmission in mice immunized with native NcSRS2 and challenged with parasites compared to mice in non-immunized groups.

An interesting finding in this study was the association of protection to *N. caninum* congenital transmission with a bias of the immune system to a Th2 response. The majority of previous studies suggest the paradigm of a Th1 immune response, characterized by relative increases in IFN-γ and IgG2a relative to IL-4 and IgG1, respectively, is necessary for protection against

neosporosis (Khan et al., 1997; Long et al., 1998; Baszler et al., 1999; Baszler et al., 2000; Ritter et al., 2002). It is possible that a Th1 response is protective primarily against encephalitic neosporosis, but is not the primary protective response against congenital transmission. However, in a previous study in our laboratory when IL-4, a Th2 cytokine, was neutralized in conjunction with pre-pregnancy infection, congenital transmission was decreased in mice subsequently challenged with virulent parasite. This suggests that a Th1 response protective against congenital transmission can be functional during pregnancy (Long and Baszler, 2000).

Alternatively, subunit immunization with native NcSRS2 may induce a protective response that overrides the permissive effects of a predominant Th2 immune response relative to parasite congenital transmission. It has been shown that with DNA immunization encoding NcSRS2 immune protection correlates with relative increase in IL 4 over IFN-γ (Nishikawa et al., 2003). It is also possible that redirection of the immune system to a primarily Th2 response is due to a combination of antigen effects and pregnancy effects (Kano et al., 2005). Similarly, our study utilizing native NcSRS2 immunization to protect against congenital transmission correlated with relative increases in IL-4 and IgG1, compared with IFN-γ and IgG2a. Since the effector function of Th2 immunity is primarily humoral, these studies suggest that induction of a protective antibody response to neosporosis is possible. There is clearly support for further investigation of *N. caninum* subunit antigens as potential vaccine antigens and for the role of protective antibody responses in prevention of *N. caninum* congenital transmission.

Acknowledgements

The author would like to thank Dr Don Knowles and Lowell Kappmeyer for their assistance in developing the immunoaffinity column used in purification of native NcSRS2. The author would also like to thank Dr Camilla Bjorkman for the SweB1 isolate of *Neospora caninum*. This research is supported by NIH KO8 Grant # 5K08HD041466 and USDA-BARD Grant # US2913-97.

References

Andrianarivo, A.G., Rowe, J.D., Barr, B.C., Anderson, M.L., Packham, A.E., Sverlow, K.W., Choromanski, L., Loui, C., Grace, A., Conrad, P.A., 2000. A POLYGEN-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following i.v./i.m. experimental tachyzoite challenge. Int. J. Parasitol. 30, 985–990.

Barber, J.S., Holmdahl, O.J., Owen, M.R., Guy, F., Uggla, A., Trees, A.J., 1995. Characterization of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper and Uggla). Parasitology 111 (Pt 5), 563–568.

Barr, B.C., Conrad, P.A., Breitmeyer, R., Sverlow, K., Anderson, M.L., Reynolds, J., Chauvet, A.E., Dubey, J.P., Ardans, A.A., 1993. Congenital *Neospora* infection in calves born from cows that had previously aborted *Neospora*-infected fetuses: four cases (1990–1992). J. Am. Vet. Med. Assoc. 202, 113–117.

Baszler, T.V., Knowles, D.P., Dubey, J.P., Gay, J.M., Mathison, B.A., McElwain, T.F., 1996. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. J. Clin. Microbiol. 34, 1423–1428.

Baszler, T.V., Long, M.T., McElwain, T.F., Mathison, B.A., 1999. Interferon-gamma and interleukin-12 mediate protection to acute *Neospora caninum* infection in BALB/c mice. Int. J. Parasitol. 29, 1635–1646.

Baszler, T.V., McElwain, T.F., Mathison, B.A., 2000. Immunization of BALB/c mice with killed *Neospora caninum* tachyzoite antigen induces a type 2 immune response and exacerbates encephalitis and neurological disease. Clin. Diagn. Lab. Immunol. 7, 893–898.

Burns, W.C., Liu, Y.S., Dow, C., Thomas, R.J., Phillips, W.A., 1997. Direct PCR from paraffin-embedded tissue. Biotechniques 22, 638–640.

Cannas, A., Naguleswaran, A., Muller, N., Eperon, S., Gottstein, B., Hemphill, A., 2003. Vaccination of mice against experimental *Neospora caninum* infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. Parasitology 126, 303–312.

Cho, J., Chung, W., Song, K., Na, B., Kang, S., Song, C., Kim, T., 2005. Protective efficacy of vaccination with *Neospora caninum* multiple recombinant antigens against experimental *Neospora caninum* infection. Korean J. Parasitol.1, 19–25.

Conrad, P.A., Barr, B.C., Sverlow, K.W., Anderson, M., Daft, B., Kinde, H., Dubey, J.P., Munson, L., Ardans, A., 1993. In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. Parasitology 106 (Pt 3), 239–249.

Debard, N., Buzoni-Gatel, D., Bout, D., 1996. Intranasal immunization with SAG1 protein of *Toxoplasma gondii* in association with cholera toxin dramatically reduces development of cerebral cysts after oral infection. Infect. Immun. 64, 2158–2166.

Dubey, J.P., 2003. Review of *Neospora caninum* and neosporosis in animals. Korean J. Parasitol. 41, 1–16.

Dubey, J.P., Carpenter, J.L., Speer, C.A., Topper, M.J., Uggla, A., 1988. Newly recognized fatal protozoan disease of dogs. J. Am. Vet. Med. Assoc. 192, 1269–1285.

Gondim, L.F., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004. Coyotes (Canis latrans) are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 34, 159–161.

Hay, W.H., Shell, L.G., Lindsay, D.S., Dubey, J.P., 1990. Diagnosis and treatment of *Neospora caninum* infection in a dog. J. Am. Vet. Med. Assoc. 197, 87–89.

Hemphill, A., 1996. Subcellular localization and functional characterization of Nc-p43, a major *Neospora caninum* tachyzoite surface protein. Infect. Immun. 64, 4279–4287.

Hemphill, A., Gottstein, B., 1996. Identification of a major surface protein on *Neospora caninum* tachyzoites. Parasitol. Res. 82, 497–504.

Howe, D.K., Crawford, A.C., Lindsay, D., Sibley, L.D., 1998. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. Infect. Immun. 66, 5322–5328.

Innes, E.A., Wright, S.E., Maley, S., Rae, A., Schock, A., Kirvar, E., Bartley, P., Hamilton, C., Carey, I.M., Buxton, D., 2001. Protection against vertical transmission in bovine neosporosis. Int. J. Parasitol. 31, 1523–1534.

Jenkins, M.C., Tuo, W., Dubey, J.P., 2004. Evaluation of vaccination with *Neospora caninum* protein for prevention of fetal loss associated with experimentally induced neosporosis in sheep. Am. J. Vet. Res. 65, 1404–1408.

Kano, R., Masukata, Y., Omata, Y., Kobayashi, Y., Maeda, R., Saito, A., 2005. Relationship between type 1/type 2 immune responses

and occurrence of vertical transmission in BALB/c mice infected with *Neospora caninum*. Vet. Parasitol. 129, 159–164.

Khan, I.A., Schwartzman, J.D., Fonseka, S., Kasper, L.H., 1997. *Neospora caninum*: role for immune cytokines in host immunity. Exp. Parasitol. 85, 24–34.

Liddell, S., Jenkins, M.C., Collica, C.M., Dubey, J.P., 1999. Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. J. Parasitol. 85, 1072–1075.

Long, M.T., Baszler, T.V., 2000. Neutralization of maternal IL-4 modulates congenital protozoal transmission: comparison of innate versus acquired immune responses. J. Immunol. 164, 4768–4774.

Long, M.T., Baszler, T.V., Mathison, B.A., 1998. Comparison of intracerebral parasite load, lesion development, and systemic cytokines in mouse strains infected with *Neospora caninum*. J. Parasitol. 84, 316–320.

Marsh, A.E., Howe, D.K., Wang, G., Barr, B.C., Cannon, N., Conrad, P.A., 1999. Differentiation of *Neospora hughesi* from *Neospora caninum* based on their immunodominant surface antigen, SAG1 and SRS2. Int. J. Parasitol. 29, 1575–1582.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.

McAllister, M.M., Bjorkman, C., Anderson-Sprecher, R., Rogers, D.G., 2000. Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. J. Am. Vet. Med. Assoc. 217, 881–887.

Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H., Mikami, T., 2000a. Monoclonal antibody inhibition of *Neospora caninum* tachyzoite invasion into host cells. Int. J. Parasitol. 30, 51–58.

Nishikawa, Y., Kousaka, Y., Fukumoto, S., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H., Mikami, T., 2000b. Delivery of *Neospora caninum* surface protein, NcSRS2 (Nc-p43), to mouse using recombinant vaccinia virus. Parasitol. Res. 86, 934–939.

Nishikawa, Y., Inoue, N., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H., Mikami, T., 2001a. Protective efficacy of vaccination by recombinant vaccinia virus against *Neospora caninum* infection. Vaccine 19, 1381–1390.

Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H., Mikami, T., 2001b. Prevention of vertical transmission of *Neospora caninum* in BALB/c mice by recombinant vaccinia virus carrying NcSRS2 gene. Vaccine 19, 1710–1716.

Nishikawa, Y., Inoue, N., Makala, L., Nagasawa, H., 2003. A role for balance of interferon-gamma and interleukin-4 production in protective immunity against *Neospora caninum* infection. Vet. Parasitol. 116, 175–184.

O'Handley, R.M., Morgan, S.A., Parker, C., Jenkins, M.C., Dubey, J.P., 2003. Vaccination of ewes for prevention of vertical transmission of *Neospora caninum*. Am. J. Vet. Res. 64, 449–452.

Quinn, H.E., Miller, C.M., Ryce, C., Windsor, P.A., Ellis, J.T., 2002. Characterization of an outbred pregnant mouse model of *Neospora caninum* infection. J. Parasitol. 88, 691–696.

Ritter, D.M., Kerlin, R., Sibert, G., Brake, D., 2002. Immune factors influencing the course of infection with *Neospora caninum* in the murine host. J. Parasitol. 88, 271–280.

Romero, J.J., Perez, E., Frankena, K., 2004. Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions. Vet. Parasitol. 123, 149–159.

Staska, L.M., Davies, C.J., Brown, Wendy C., McGuire, Travis C., Suarez, Carlos E., Park, Joo Youn, Mathison, Bruce A., Abbott, Jeffrey R., Baszler, Timothy V., 2005. Vaccine-candidate peptides in the NcSRS2 surface protein of *Neospora caninum* identified by CD4+ CTL and IFN-ã-secreting T lymphocytes of infected Holstein cattle. Infect. Immun. 73, 1321–1329.

Stenlund, S., Bjorkman, C., Holmdahl, O.J., Kindahl, H., Uggla, A., 1997. Characterization of a Swedish bovine isolate of *Neospora caninum*. Parasitol. Res. 83, 214–219.

Williams, D.J., Guy, C.S., Smith, R.F., Guy, F., McGarry, J.W., McKay, J.S., Trees, A.J., 2003. First demonstration of protective immunity against foetopathy in cattle with latent *Neospora caninum* infection. Int. J. Parasitol. 33, 1059–1065.

Yamage, M., Flechtner, O., Gottstein, B., 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain 'cyst' DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J. Parasitol. 82, 272–279.

Yamane, I., Shibahara, T., Kokuho, T., Shimura, K., Hamaoka, T., Haritani, M., Conrad, P.A., Park, C.H., Sawada, M., Umemura, T., 1998. An improved isolation technique for bovine *Neospora species*. J. Vet. Diagn. Invest. 10, 364–368.